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Ectoparasitic arthropods are often vectors of rickettsiosis. We conducted a survey of ectoparasites on U.S. military facilities throughout Japan with the use of specimens submitted by pest control, public health, and veterinary personnel. Over 1,600 individual ectoparasites were collected. Fifteen species were identified, including several significant vectors of human diseases such as scrub typhus and rickettsial spotted fevers. These ectoparasites included Ctenocephalides felis, Haemaphysalis longicornis, Ixodes persulcatus, Leptotrombidium fuji, Leptotrombidium pallidum, and Rhipicephalus sanguineus. Rickettsial agents were detected by PCR and DNA sequencing. These included Bartonella henselae, Bartonella japonica, a novel Bartonella, Coxiella burnetii, an unnamed Coxiella, Ehrlichia canis, Orientia tsutsugamushi, Rickettsia typhi, and ''Rickettsia Rf2125''/''Rickettsia cf1and5''.

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ABSTRACT: Ectoparasitic arthropods are often vectors of rickettsiosis. We conducted a survey of ectoparasites on U.S. military facilities throughout Japan with the use of specimens submitted by pest control, public health, and veterinary personnel. Over 1,600 individual ectoparasites were collected. Fifteen species were identified, including several significant vectors of human diseases such as scrub typhus and rickettsial spotted fevers. These ectoparasites included *Ctenocephalides felis, Haemaphysalis longicornis*, *Ixodes persulcatus*, *Leptotrombidium fuji*, *Leptotrombidium pallidum*, and *Rhipicephalus sanguineus*. Rickettsial agents were detected by PCR and DNA sequencing. These included *Bartonella henselae*, *Bartonella japonica*, a novel *Bartonella*, *Coxiella burnetii*, an unnamed *Coxiella*, *Ehrlichia canis*, *Orientia tsutsugamushi*, *Rickettsia typhi*, and "*Rickettsia* Rf2125"/"*Rickettsia* cf1and5".

Rickettsioses have historically plagued military forces and continue to be a threat (Kelly et al., 2002). With the exception of epidemic typhus, most of these diseases are zoonotic, humans are dead-end hosts, and human exposure is sporadic. Several rickettsial agents are geographically restricted, infect only a few vertebrate hosts, or are transmitted by a limited range of vectors; whereas others are more cosmopolitan, infect a wide range of vertebrates, and are transmitted by numerous vectors.

United States Forces–Japan has geographically separated bases managed by different services (Fig. 1); however, there is a common threat from vector-borne diseases. Zoonotic pathogens are present on U.S. bases (Reeves et al., 2013). We describe the results of an integrated rickettsial pathogen surveillance program in Japan that incorporated arthropod sample submission from public health officers, entomologists, pest management personnel, and veterinary staff at regional clinics. We summarize pathogen and ectoparasite surveillance from May 2009 through February 2011. An accurate understanding of ectoparasite-borne pathogens is critical for both control measures and for medical doctors or veterinarians diagnosing disease. Medical entomologists and pest control personnel often make their threat assessments or control decisions based on published information, medical histories, or geographically relevant vector or pathogen distributions. Most medical staff on military bases are present for only a few years and can only make diagnoses of vector-borne diseases that they are aware of in the literature or that were reported by medical entomologists. New or previously poorly recorded ectoparasite and pathogen associations allow reassessments of risk or novel threat assessments. Some ectoparasites of wildlife never directly contact humans but could be significant for transmission of pathogens between animals.

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MATERIALS AND METHODS

Ectoparasites were collected by hand from Department of Defense personnel, on animals at veterinary clinics, or from dead animals trapped by pest control operators on bases. Ectoparasites were identified with the use of the taxonomic keys for Japanese fleas (406th Medical Laboratory, 1953; Sakaguti and Jameson, 1962) and ticks and mites (Yamaguti et al., 1971; Ehara, 1980) or by comparison with reference materials.

Following identification, each ectoparasite was macerated with a sterile razor blade and a Teflon pestle before the remains were digested with Proteinase K. Chiggers and listrophorid mites were pooled for DNA extraction and testing. Total DNA was extracted from individual or pooled ectoparasites (Table I) with a DNeasy Blood & Tissue Kit (Qiagen, Valencia, California) and resuspended in nuclease-free water. Extracts were screened for DNA from *Anaplasma*, *Bartonella*, *Coxiella burnetii*, *Ehrlichia*, *Orientia tsutsugamushi*, and *Rickettsia* by polymerase chain reaction (PCR). We followed the real-time PCR protocols described by Loftis et al. (2006) for *Anaplasma*, *Bartonella*, *Coxiella burnetii*, *Ehrlichia*, and *Rickettsia* and by Jiang et al. (2004) for *O. tsutsugamushi*. Controls for each assay included distilled water as a negative control and synthetic DNA oligonucleotides that corresponded to the primers and probe but were unique. The synthetic DNA oligonucleotides served as an additional control, because they could be differentiated from real agents by DNA sequencing. Positive samples were further characterized by sequencing DNA from additional traditional PCR amplicons. We amplified DNA from the 17 kD antigen gene of *Rickettsia* with the use of Primer-1 and Primer-2 (Webb et al., 1990), the 16S rRNA gene of *Anaplasma* and *Ehrlichia* with the use of the EC12A and HE3 primers (Reeves et al., 2005), the *gltA* gene of *Bartonella* with the BhCS.781 and BHCS.1137 primers (Norman et al., 1995), and the 56 kD antigen gene of *O. tsutsugamushi* with nested PCR as described by Horinouchi et al. (1996). In addition, we amplified the bacterial 16S rRNA gene from some positive samples with the RickF1 and RickR4 primers described by Reeves (2005). PCR products were separated by electrophoresis on 4% agarose gels and visualized with ethidium bromide under ultraviolet light. Products were purified with a QIAquick PCR Purification Kit (Qiagen). Sequencing reactions were performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) with the use of PCR primers, and excess dye was removed by ethanol precipitation. Sequences were determined with the use of an ABI 3700 capillary sequencer (Applied Biosystems), aligned and assembled with Chromas Lite 2.01 (Technelysium, Queensland, Australia) and ClustalW (Kyoto University Bioinformatics Center, Kyoto, Japan), and compared to sequences in GenBank with the use of the BLAST 2.0 program (NCBI, Bethesda, Maryland). Novel DNA sequences of the *gltA* gene from *Bartonella* detected in listrophorid mites were deposited in GenBank with the accession number HM006916. All other sequences were identical to 1 or more GenBank accession and were not resubmitted.

RESULTS

A wide range of ectoparasites was collected on bases (Table I). Some of these are known vectors of significant pathogens. These included 2 species of chiggers, *Leptotrombidium pallidum* and *Leptotrombidium fuji*, from rodents trapped on Camp Zama. Both

FIGURE 1. Map of Japan showing U.S. military bases where sampling was conducted.

are potential vectors of the causative agent of scrub typhus, *O. tsutsugamushi* (Tamura et al., 2000). In addition, several medically significant species of ticks were collected. These included *Haemaphysalis longicornis*, a vector of *Rickettsia japonica,* the agent of Japanese spotted fever (Uchida et al., 1995); and *R. sanguineus*, a nearly cosmopolitan ectoparasite of dogs that is a vector of several disease agents including those that cause anaplasmosis, babesiosis, ehrlichiosis, rickettsial spotted fevers, Q-fever, and hepatozoonosis (Shaw et al., 2001). *Ixodes persulcatus*, a vector of tick-borne encephalitis virus and other pathogens in Japan (Takashima, 1998) was collected on Camp Zama. Fleas and lice were collected from both wild and domestic animals (Table I). Most of these are ectoparasites of wildlife and have little possibility of coming into direct contact with humans. One exception is the cat flea, *Ctenocephalides felis*, a vector of a filarial nematode of dogs and rickettsial agents such as *Bartonella* and *Rickettsia* spp. (Foil et al., 1998; Wedincamp and Foil, 2002). All ectoparasites were tested by PCR, and 9 different rickettsial agents were detected, including *Bartonella henselae*, *Bartonella japonica*, a novel *Bartonella*, *Coxiella burnetii*, an unnamed *Coxiella* of ticks, *Ehrlichia canis*, *O. tsutsugamushi*, *Rickettsia typhi*, and "*Rickettsia* Rf2125"/"*Rickettsia* cf1and5".

DISCUSSION

Many ectoparasites of wildlife, such as lice and mites, are poorly studied or rarely associated with pathogens. These ectoparasites are unlikely to come into contact with humans but they can serve to maintain pathogens enzootically in wildlife populations. Military working dogs are treated chemoprophylactically to control ticks, lice, and fleas. However, they can still be exposed to ectoparasites. We have several samples of ticks from working dogs in the Pacific Theater outside of Japan (unpubl. data). Ectoparasite control can reduce exposure risk but does not eliminate it.

Several vector-borne rickettsial agents were detected in ectoparasites from the military bases (Table I). We briefly discuss the relevance of each agent. Rickettsial diseases in humans and military dogs are infrequently reported (e.g., Anna et al., 2012) and military deaths from rickettsioses are rare (Reeves and Bettano, 2014). Some of these agents cause self-limiting febrile illnesses that are likely to be unidentified. Actual work time lost from vector-borne rickettsial agents on military bases in Japan is unknown. Some of the pathogens detected were well characterized and known to cause disease, whereas others were novel or remain poorly studied.

Cat scratch disease is among the most frequently reported rickettsial disease in the United States, with over 20,000 cases recorded annually (Kaplan et al., 2002). The disease is rarely fatal but can cause permanent heart damage in some individuals (Zeaiter et al., 2003). *Bartonella henselae*, the agent of cat scratch disease, was detected in fleas removed from a cat on Camp Zama and a stray cat from Atsugi Naval Air Base. Prior to adoption, the animal was treated for fleas and given antibiotics while at the clinic. In addition, 5 nymphs of *Haemaphysalis japonica* from a peridomestic tanuki, *Nyctereutes procyonoides viverrinus*, tested positive for *B. henselae*. There is growing evidence that ticks might play a role in the transmission of *Bartonella* spp. in Asia (Kim et al., 2005). A blood sample from the tanuki was negative for *B. henselae*. It could have had a low bacteremia or the ticks could have been infected from a previous blood meal. Detection of *B. henselae* from wildlife and feral animals on base housing areas indicate that this agent is circulating in the environment.

The *gltA* gene from a novel *Bartonella* was amplified from 3 pools of unidentified listrophorid mites removed from Japanese moles, *Mogera imaizumii* (Table I). The DNA sequences were identical to each other and 84% similar to the *gltA* gene of *Bartonella clarridgeiae* (GenBank GU056189). DNA from *Bartonella* was not amplified from the moles or any of their other ectoparasites. The amplification of DNA from *Bartonella* from samples collected in different traps, locations, and dates indicates this was not an erroneous detection. The data indicate that *Bartonella* is associated with listrophorid mites. The pathogenicity and transmission of this novel *Bartonella* is unknown. Listrophorid mites are associated with hair, skin, and sebaceous materials and could be shedding *Bartonella*, but these mites do not bite their hosts (OConnor, 2009). Further research is needed to determine if other listrophorid mites throughout the world harbor *Bartonella* spp. or if these mites play a role in the transmission of pathogens. We were unable to find any published literature where *Bartonella* was detected in listrophorid mites. These mites are largely ignored as potential vectors. There is very little published on the ectoparasites of *M. imaizumii*. Some of the mites collected on Camp Zama, Japan included a new species of *Oryctolaelaps* that will be described elsewhere. The listrophorid mites were also possibly unnamed.

Bartonella japonica was detected in 2 pools of rodent lice, *Hoplopleura affinis*. *Bartonella japonica* was originally described from field mice (*Apodemus* spp.) trapped in the forests around Mt. Fuji, Japan (Inoue et al., 2010). There were no known vectors for this *Bartonella* (Inoue et al., 2010). Based on the molecular association between a *H. affinis* and *B. japonica* further research could focus on louse-borne transmission of the pathogen. Sucking lice transmit some *Bartonella* spp. including *Bartonella quintana* (Alsmark et al., 2004). *Bartonella japonica* is not known to infect

TABLE I. Collection data for ectoparasites from U.S. military bases in Japan and associated pathogens from 2009–2011.

Location	Species	Date collected	No.	Host	Agent (number positive)
Camp Zama	Astigmatid mite	31 March to 30 April 2010	220	*Mogera imaizumii*	None
Atsugi Naval Air Station	*Ctenocephalides felis*	1 September 2009	7	*Felis catus*	*Bartonella henselae* (3)
Camp Zama	*C. felis*	3 September 2009	1	*F. catus*	None
Camp Zama	*C. felis*	1 September 2010	2	*F. catus*	*B. henselae* (1)
Camp Zama	*C. felis*	30 June 2009	1	*F. catus*	*Rickettsia typhi* (1)
Camp Zama	*C. felis*	15 July to 19 Nov 2009	6	*Canis lupus* (dog)	"*Rickettsia* cf1and5" (1)
Camp Zama	*C. felis*	19 Nov 2009 to 13 September 2010	7	*F. catus*	"*Rickettsia* cf1and5" (4)
Iwakuni Marine Corps	*C. felis*	27 July 2010	16	*Canis lupus* (dog)	"*Rickettsia* cf1and5" (16)
Kadena Veterinary Clinic	*C. felis*	23 March 2010	1	*F. catus*	None
Sagami Housing	*C. felis*	19 September 2009 to 22 October 2010	17	*F. catus*	"*Rickettsia* cf1and5" (5)
Sagami Housing	*C. felis*	18 September 2009	5	*C. lupus* (dog)	None
Sagami Housing	*C. felis*	31 September 2009	4	*Procyon lotor*	None
Camp Zama	*Eulaelaps stabularis*	6 January 2010	10	*Rattus norvegicus*	None
Camp Zama	Feather mite	29 January 2010	10	*Carduelis sinica*	None
Atsugi Naval Air Station	Feather mite	29 January 2010	13	Unknown bird	None
Camp Zama	*Haemaphysalis* sp.	5 July 2009	1	*Homo sapiens*	None
Iwakuni Marine Corps	*Haemaphysalis flava*	21 June 2010	5	*C. lupus* (dog)	*Coxiella* sp. (5)
Camp Hanson	*Haemaphysalis ias*	23 May 2009	14	*C. lupus* (dog)	None
Sagami Housing	*Haemaphysalis japonica*	24 August 2010	2	*C. lupus* (dog)	None
Sagami Housing	*H. japonica*	31 September 2009	1	*P. lotor*	None
Sagami Housing	*H. japonica*	3 January 2010	4	*Nyctereutes procyonoides viverrinus*	None
Camp Zama	*H. japonica*	30 September 2009	9	*N. p. viverrinus*	*B. henselae* (5)
Sagami Housing	*Haemaphysalis longicornis*	3 January 2010	1	*C. lupus* (dog)	None
Camp Zama	*Hoploplura affinis*	16 October 2009 to 20 May 2010	25	*Apodemus speciosus*	*Bartonella japonica* (5)
Camp Zama	*Ixodes persulcatus*	29 January 2010	9	*C. sinica*	None
Camp Zama	Laelapidae	15 September 2009 to 23 February 2010	44	*A. speciosus*	None
Camp Zama	Chigger	11 January 2010	3	*M. imaizumii*	None
Camp Zama	*Leptotrombidium* spp.	30 September 2009 to 2 October 2009	12	*A. speciosus*	None
Camp Zama	*Leptotrombidium pallidum*	29 September to 16 October 2009	79	*A. speciosus*	*Orientia tsutsugamushi* (1 pool)
Camp Zama	*L. pallidum* and *Leptotrombidium fuji*	6 January 2010	200+	*R. norvegicus*	*O. tsutsugamushi* (1 pool)
Camp Zama	Listrophoridae	11 January 2010 to 17 February 2010	510	*M. imaizumii*	*Bartonella* n. sp. (3 pools)
Camp Zama	Mesostigmatid mite	29 January 2010	9	*C. sinica*	None
Camp Zama	Mesostigmatid mite	3 June 2010	7	*Hirundo rustica*	None
Camp Zama	*Myocoptes* sp.	6 January 2010	1	*R. norvegicus*	None
Camp Zama	*Neopsylla sasai*	26 October 2009 to 23 February 2010	1	*A. speciosus*	None
Camp Zama	*Ornithonyssus bacoti*	29 July 2009	11	*R. norvegicus*	None
Camp Zama	*O. bacoti*	17 August 2009	50	*Mus musculus*	None
Camp Zama	*Oryctolaelaps* n. sp.	11 January 2010 to 7 April 2010	89	*M. imaizumii*	None
Camp Zama	*Polyplax serrata*	17 August 2009	2	*M. musculus*	None
Camp Zama	*P. serrata*	15 September to 15 October 2009	23	*A. speciosus*	None

Table I. Continued.

Location	Species	Date collected	No.	Host	Agent (number positive)
Camp Zama	*Polyplax spinulosa*	29 July 2009	5	*R. norvegicus*	None
Kadena Ona Point	*Rhipicephalus sanguineus*	25 August 2009	5	*C. lupus* (dog)	None
Kadena Veterinary Clinic	*R. sanguineus*	23 March 2010	170	*C. lupus* (dog)	*Coxiella burnetii* (1) and *Ehrlichia canis* (1 and 1 weak positive)
Camp Hanson	*R. sanguineus*	23 May 2009	1	*C. lupus* (dog)	None

humans or domestic animals. Sucking lice (*Hoplopleura pacifica, Hoplopleura akanezumi,* and *Polyplax spinulosa*) of murine rodents in Japan (*Rattus rattus* and *Apodemus speciosus*) have been reported to be capable of experimentally transmitting the rickettsial agents of scrub typhus, murine typhus, and epidemic typhus under laboratory conditions (Kaneko, 1959).

Rickettsia typhi, the agent of murine typhus, was detected in a single cat flea, *C. felis*. The cat flea is a potential vector of *R. typhi* (Azad, 1990; Fergie et al., 2000), but the relevance of cat fleas is not fully understood. *Xensopsylla cheopis*, a rat flea, is the classic vector of murine typhus (Azad, 1990). Murine typhus is a public health problem in Asia that is often overlooked or misdiagnosed (Parola et al., 2003a), and its continued presence should be noted by health-care providers. The infected flea was collected from a pet living in housing with a continuous flea infestation problem. After detection, public works initiated a rodent and feral animal control program in the area to reduce the threat from flea-borne rickettsiae.

An unnamed *Rickettsia* was detected in several *C. felis*. Specimens of *C. felis* harboring this agent were collected from both cats and dogs (Table I). The DNA sequence was a 100% match to GenBank sequences named "*Rickettsia* cf1and5". This is a *Rickettsia* of unknown pathogenicity if it can even infect vertebrates. The *Rickettsia* was originally detected in *Ctenocephalides* spp. from Thailand as "*Rickettsia* Rf2125" (Parola et al., 2003b). It was later reported from *Echidnophaga gallinacea* (the sticktight flea) and *C. felis* from Egypt, American Samoa, the Marshall Islands, and the United States (Reeves et al., 2005, 2012; Loftis et al., 2006; Tuten et al., 2013). Obhiambo et al. (2014) discuss the problems of confusing these *Rickettsia felis*–like organisms from each other. Neither human nor animal infections are known, but the agent is prevalent in fleas, so human exposure is possible. Lai et al. (2014) describe cases of rickettsioses in Taiwan and suggest that some of the *R. felis* infections could be *R. felis*–like organisms that are not fully characterized. Labruna and Walker (2014) suggest that *R. felis*–like organisms are not pathogenic and might even be symbionts of vertebrates. Other flea-borne agents such as *R. felis* were discovered and later linked to disease in humans worldwide (Reif and Macaluso, 2009). Further research is needed to determine if there are serologic conversions in humans or animals (Lai et al., 2014).

A brown dog tick (*Rhipicephalus sanguineus*) from an Okinawan dog tested positive for *Ehrlichia canis*. Inokuma et al. (1998) presented serologic evidence of *E. canis* in dogs from Okinawa. *Ehrlichia canis* is a potential threat to military working dogs so continued vigilance in tick control is important. A tick collected at the same time from the same animal was real time PCR positive for either an *Ehrlichia* or *Anaplasma* but the CT value was 34 and we were unable to amplify DNA from this tick with the use of conventional PCR. It possibly harbored *E. canis,* from an infected blood meal but an infection with *Anaplasma platys* or some other agent could be involved.

Scrub typhus (caused by *O. tsutsugamushi*) is a significant threat to public health throughout much of Asia. It is transmitted by chiggers, which are ectoparasitic larval mites (Family Trombiculidae). Most of the chiggers collected during surveillance were removed from the ears of rodents. The majority of chiggers were not identified, because the DNA extraction protocol was destructive. However, selected specimens from each host were slide mounted and identified as *Leptotrombidium pallidum* and *Leptotrombidium fuji*.

Two pools of chiggers from a rat trapped on Camp Zama were PCR positive for *O. tsutsugamushi*. Both pools of chiggers were from rats trapped in the scrub habitat near the base golf course. Camp Zama is in Kanagawa Prefecture and significant epidemics of scrub typhus occur in this prefecture in unpredictable cycles (Furuya et al., 2000; Katayama et al., 2006). The presence of *O. tsutsugamushi* in chiggers was not surprising and should serve as a reminder to golfers and maintenance personnel to use appropriate protection against chiggers. We were unable to survey Camp Fuji, but there were scrub typhus cases in the U.S. Marines training there in 2000 and 2001 (Jiang et al., 2003).

Two species of *Coxiella* were detected in ectoparasites. *Coxiella burnetii* was detected in brown dog ticks from Okinawa. This is the agent of Q-fever and it has been frequently associated with ticks. Porter et al. (2011) recently reviewed the distribution of Q-fever in Japan. Andoh et al. (2013) also surveyed for *C. burnetii* in Japanese ticks. They looked at ticks from 15 dogs in Okinawa and did not find any with *C. burnetii*, however Andoh et al. (2013) noted that 10–15% of dogs in some serosurveys in Japan were antibody positive. A second unnamed *Coxiella* endosymbiont of *Haemaphysalis* was detected in 1 pool of ticks. The DNA from this *Coxiella* was identical based on those already reported by Lee et al. (2004). Qui et al. (2014) reported these *Coxiella* are in the salivary glands of some ticks and thus could be transmitted to vertebrate hosts.

We were unable to detect DNA from *Anaplasma* spp. in any of the samples. Although our samples were negative, *Anaplasma phagocytophilum* was reported from Japanese ticks (Ohashi et al.,

2005). A novel *Anaplasma* sp. was recently reported from ticks in northern Japan (Ybañez et al., 2013).

Ectoparasites and associated rickettsiosis pose an ongoing threat to military personnel throughout Japan. Our surveillance detected both potential vectors and pathogens on several bases. Rickettsial diseases in humans are relatively rare, because exposure to infected vectors is sporadic and infrequent. Clinicians should be aware that these pathogens are present on bases and that the associated diseases can be misidentified. For example, scrub typhus can present with signs of acute rapid onset deafness (Premaratna et al., 2006). Spotted fevers can resemble other diseases such as arboviral infections (Premaratna et al., 2011). Continued vigilance is needed both to protect patients and to survey and control the vectors of these diseases.

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